약학박사 학위논문

Evaluation of N,N,N-trimethylphytosphingosine-iodide and its liposomal delivery system for the treatment of angiogenesis and metastasis

혈관형성과 암전이 치료를 위한 N,N,N-트리메틸피토스핑고신 및 리포솜 전달 시스템의 평가

2014 년 2 월

서울대학교 대학원

제약학과 약제과학 전공

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이 논문을 약학박사 학위논문으로 제출함

2013 년 11 월

서울대학교 대학원

제약학과 약제과학 전공

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송충길의 약학박사 학위논문을 인준함

2013 년 12 월

위 원 장 _____(인)

부위원장 _____(인)

위 원 _____(인)

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ABSTRACT

Evaluation of N,N,N-trimethylphytosphingosine-iodide and its liposomal delivery system for the treatment of angiogenesis and metastasis

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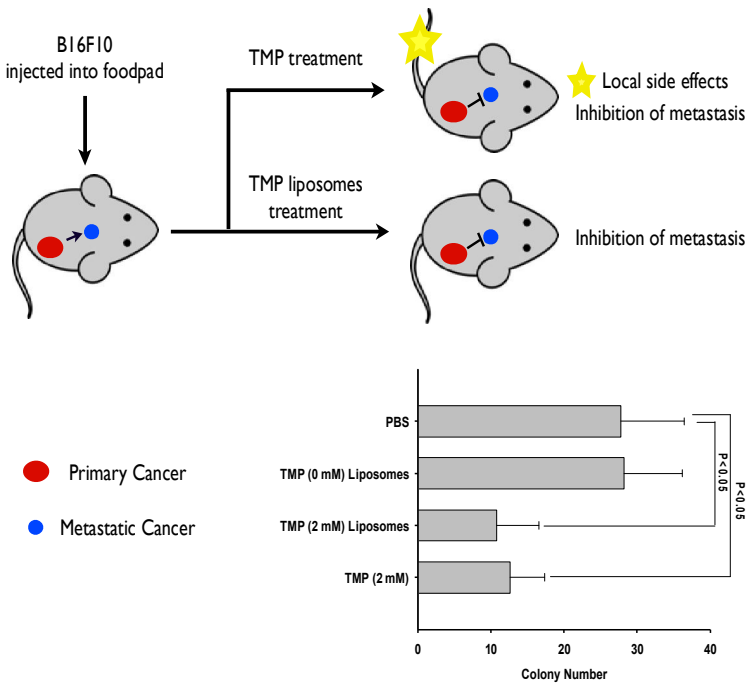
Phytosphingosine and methyl derivatives are important mediators on cellular processes, and are associated with cell growth and death. The antitumor activity of *N,N,N*-trimethylphytosphingosine-iodide (TMP) as a novel potent inhibitor of angiogenesis and metastasis was evaluated in B16F10 murine melanoma cells. The results indicated that TMP itself effectively inhibited *in vitro* cell migration, tube formation, and the expression of angiogenic factors as well as *in vivo* lung metastasis. However, TMP slightly suppressed *in vivo* experimental tumor metastasis in its free form and induced side effects including hemolysis and local side effects. Therefore, in an attempt to reduce the toxicity and the undesirable side effects of TMP, a liposomal formulation was prepared and tested for its effectiveness. TMP liposomes retained the

effectiveness of TMP *in vitro* while side effects were reduced, and both *in vivo* experimental and spontaneous tumor metastasis were significantly suppressed. These results support the conclusion that TMP effectively inhibits *in vitro* angiogenesis as well as *in vivo* metastasis, and a liposomal formulation is more efficient delivery system for TMP treatment than solution.

Keywords: *N,N,N*-trimethylphytosphingosine-iodide; liposomes; angiogenesis; metastasis

Student Number: 2007-30462

GRAPHIC ABSTRACT



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1. Introduction

Metastasis is a word of greek origin meaning “displacement”, from μετά (meta, "next") and στάσις (stasis, "placement"). Metastases are the main cause of death in patients with cancer. Metastases arise following the spread of cancer from a primary site and the formation of new tumors in adjacent or non-adjacent organs (Fig. 1). The metastatic process consists of a series of steps all of which must be successfully completed to give rise to a metastatic tumor (Woodhouse et al., 1997). Cancer metastasis is required the growth of a new network of blood supply, called “angiogenesis”, which new blood vessels are formed from pre-existing vessels for wound repair, the development of granulation tissue and tumors (Folkman, 1971; Ossowski and Reich, 1983). These new blood vessels can also provide an escape route by which cells can leave the tumor and enter into the body’s circulatory blood system known as intravasation (Wyckoff et al., 2000). Tumor cells might also infiltrate the blood circulatory system indirectly via the lymphatic system. The cells need to survive in the circulation until they can arrest in a new organ; here, they might extravasate from the circulation into the surrounding tissue. Once in the new site, cells must initiate and maintain growth to form pre-angiogenic micrometastases. Various studies dealing with tumor angiogenesis have pointed out that tumor expansion with the formation of new blood vessels and tumor regression due to angiogenic inhibitors are correlated with metastasis (Holash et al., 1999; Hood et al., 2002; Weidner et al., 1991, 1993). During the process of tumor angiogenesis, highly permeable blood vessels are formed in and

around solid tumors, thus providing an efficient translocation route for tumor cells to leave the primary tumor site, possibly inducing metastasis (Zetter, 1998). Therefore the use of angiogenic inhibitors for the suppression of metastasis has been an area of considerable interest (Ebos et al., 2009; Konno et al., 1995; O'Reilly et al., 1994; Pàez-Ribes et al., 2009).

Sphingolipids, which were discovered in brain extract in the 1870s, are a class of lipids containing the organic aliphatic amino alchol shingosine or a substance structurally similar to it. Sphingolipids and their derivatives, such as ceramide, sphingosine, sphingosine-1-phosphate (S1P) and phytosphingosine, have been identified as important mediators of cellular processes, in particular, cell proliferation, differentiation, senescence and apoptosis (Merrill, 2002; Spiegel and Merrill Jr, 1996; Spiegel and Milstien, 2002; Woodcock, 2006). For instance, it has been reported that sphingosine (SPN) is a negative modulator of transmembrane signaling through protein kinase C (PKC) as well as an inhibitor of sphingosine kinase-1 (SK-1), which is implicated in cell growth and inhibitory apoptosis (Cuvillier and Levade, 2001; Hannun and Bell, 1989). Previous reports showed that PKC inhibitors could prevent the enhancement in VEGF expression plays (Xu et al., 2008). In addition, the inhibitory effect of SPN derivatives on metastatic potential has also been reported (Okoshi et al., 1991). Interestingly, *N,N,N*-trimethylsphingosine (TMS) showed a much stronger inhibitory effect on PKC activity than *N,N*-dimethylsphingosine (DMS) and unsubsitituted SPN (Endo et al., 1991). Phytosphingosine (FIG. 1A), which is similar in structure to sphingosine, is a major component of membranes produced by plants, fungi, mammalian tissue and some types of cancer cells (Jo et al., 2003). The inhibitory effects

of synthetic phytosphingosine derivatives (*N*-monomehtylphytosphingosine and *N,N*-dimehtylphytosphingosine) on SK-1 activity were recently reported to be stronger than that of DMS (Park et al., 2010). Moreover, phytosphingosine and its methyl derivatives induce the apoptotic cell death of cancer cells through ROS generation, caspase activation and Bax translocation (Kim et al., 2009; Park et al., 2003; Park et al., 2010). However, the anti-angiogenic and metastatic roles of phytosphingosine and its methyl derivatives are largely unknown in spite of their potential as anticancer cellular modulators. Clinical studies of SPN derivatives indicate that they have a number of undesirable side effects such as hemolysis, hemoglobinuria, and inflammation at the injection sites, but that they also have potent inhibitory effects on tumor growth and metastasis (Endo et al., 1991). To reduce magnitude of the side effects and enhance the activities of SPN derivatives in *in vivo* systems, the use of liposomes that contain SPN derivatives as drug delivery carriers have been proposed (Park et al., 1994). It is well known that, when liposomes are used as carriers, the systemic circulation time is prolonged and drug accumulation in cancer tissue is increased, either through surface modification or lipid composition of liposomes, and that the side effects of a drug are reduced (Gabizon et al., 1982, 1995; Matsuo et al., 2001; Song et al., 2009).

N,N,N-trimethylphytosphingosine-iodide (TMP, Fig. 1B) was recently prepared as an antitumor agent (Namgoong and Park, 2003). The objective of this study was to evaluate the *in vitro* anti-angiogenic and *in vivo* anti-metastatic activities of TMP. In addition, the mechanism of its action was investigated. However, when TMP was used in *in vivo* systems in preliminary experiments, it was observed to cause side effects similar to TMS (data not shown). Therefore, a liposomal formulation was prepared, in an attempt

to reduce the toxicity and undesirable side effects of TMP. The findings herein indicate that TMP is a potent inhibitor of angiogenesis and metastasis, and that liposomes that contain TMP retain these properties while reducing side effects during *in vivo* treatment.

2. Materials and methods

2.1 Materials

Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). *N,N,N*-trimethylphytosphingosine-iodide (TMP) was synthesized and donated by the Phytos Co. (Suwon, South Korea) (Fig. 2) (Namgoong and Park, 2003). Annexin V-FITC Apoptosis Detection Kit was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Non-radioisotopic kit for measuring protein kinase C activity was purchased from CycLex® Co. (Nagano, Japan). For cell culture, Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM) and RPMI Medium 1640 (RPMI1640) and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, NY, USA). All other materials were used without further purification.

2.2 Synthesis of *N,N,N*-trimethylphytosphingosine-iodide (TMP)

Phytosphingosine (300 mg, 0.946 mmol), iodomethane (0.298 ml, 4.73 mmol) and K_2CO_3 (523 mg, 3.79 mmol) as a protector of hydroxyl groups were dissolved in 3 ml of methanol, and the mixture was stirred for 4 h at 50°C. The solvent was evaporated under reduced pressure and 4 ml of distilled water was added to the resulting mixture. The solution was extracted with 8 ml of ethyl acetate, dried over Na_2SO_4 and filtered. The

ethyl acetate was evaporated to give 260 mg of a white solid. The chemical structure and mass of TMP were confirmed by FT-IR, ^{1}H NMR, ^{13}C NMR and MS (FAB, Glycerol, m/z) spectra. The yield of product was 76 % and the material was stored at −20°C until used. IR (KBr) υ max: 3009 (OH), 2918, 2850 (C-H) cm-1. ^{1}H NMR (600 MHz, DMSO-d_6): δ3.95 (dd, 1H, CH_2O, J=14.4 Hz), 3.89 (dd, 1H, CH_2O, J=14.4 Hz), 3.76 (d, 1H, J=8.7 Hz), 3.6 (dd, 1H), 3.11 (s, 9H, $N^{+}CH_3$), 1.68 (m, 1H, CH_2), 1.48 (m, 1H, CH_2), 1.23 (s, 24H, CH_2), 0.84 (t, 3H, CH_3) ppm. ^{13}C NMR (600 MHz, DMSO-d_6): δ76.80, 71.01, 55.69, 52.18, 33.21, 31.21, 30.60, 29.15, 29.03, 28.99, 28.93, 28.62, 24.87, 22.00, 13.83 ppm. MS (FAB) Calcd for TMP: 361 [M^{+}]) (Namgoong and Park, 2003).

2.3 Preparation and characterization of liposomes

The liposomes were composed of DPPC, CHOL (1:1 molar ratio) and various concentrations of TMP (from 0 to 400 μM). The liposomes were prepared by the thin film hydration method. Briefly, the lipids were dissolved in chloroform, and the solvent subsequently removed under reduced pressure at 50°C, using a rotary evaporator (Buchi Rotavapor R-200, Switzerland). The resulting lipid film was hydrated with phosphate-buffered saline (pH 7.4) and then gently mixed. Each liposomal solution was extruded 5 times through a 200 nm polycarbonate filter, followed by five extrusions through a 100 nm polycarbonate filter (Whatman, USA) using an extruder (Northern Lipids Inc., USA). The particle size and zeta potential of the liposomes were determined by ELS-Z Electrophoretic light scattering particle size and zeta potential analyzer

(ELS-Z, OTSUKA Electronics Co. Ltd., Japan) at room temperature. The particle size, poly dispersity and zeta potential of the TMP liposomes are shown in Table I.

2.4 *In vitro* cytotoxicity studies

Cell viability, after the addition of TMP and TMP liposomes, was determined by using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-2H-tetrazolium bromide) assay (Mosmann, 1983). Briefly, 5×10^3 cancer cells were cultured in 96-well flat-bottomed microtiter plates for 24 h at 37°C in order to evaluate the cytotoxicity of TMP or TMP liposomes. Human cancer cell lines (HepG2, MCF7, A549 and A375P) and murine cancer cell line (B16F10) were used in this experiment. The medium was replaced with serum-free medium containing various concentrations of TMP or TMP liposomes, and subsequently cultured for 24 h. And then, MTT (5 mg/ml, 20 μl) was added to each well and the cells were incubated for another 4 h at 37°C in a humidified 5% CO_2 atmosphere. A 190 μl aliquot was removed from each well, and then 150 μl of DMSO was added to solubilize the cells. Cell viabilities were then determined using a microplate reader (Emax, Molecular devices, Sunnyvale, CA) at 560 nm.

2.5 Assessment of apoptosis by FITC-Annexin V/PI staining

Double staining procedure for Annexin V and PI in B16F10 cells was slightly modified as follows previous report (Vermas et al., 1995). Briefly, 5 x 10^6 cells were resuspended in binding buffer and each FITC-Annexin V and PI was added to a final

concentration of 1 μg/ml cell suspension. The mixture was incubated for 10 min in the dark at room temperature and the cells were analyzed using a Becton Dickinson FACS and with CellQuest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA).

2.6 Hemolytic effect of TMP or TMP liposomes

The hemolytic effect of TMP or TMP liposomes in rat blood was determined based on the increased absorbance due to the release of hemoglobin from lysed erythrocytes (Park et al., 1994; Yamaji et al., 1998). Briefly, 500 μl of fresh rat whole blood treated with heparin was incubated with various concentrations of TMP (from 0 to 2 mM) or TMP liposomal solutions at 37°C for 30 min. After incubation, the mixture was centrifuged at 500 × *g* for 5 min to remove erythrocytes. An aliquot (200 μl) of supernatant was collected, and the absorbance due to the hemoglobin released from lysed erythrocytes was measured using a microplate reader (Emax, Molecular devices, Sunnyvale, CA) at 405 nm. Total hemoglobin concentration as reference was determined after freezing and thawing a sample of whole rat blood.

2.7 *In vitro* cell migration studies

The inhibition of cell migration was measured by using similar methods previously (Decaestecker et al., 2007; Liang et al., 2007). Briefly, B16F10 cells (1×10^5 cells/well) were cultured on 24 well plates at 37°C in a humidified 5% CO_2 atmosphere until 90% confluency was reached. The cells were washed with PBS and a scratch was

made on the cell monolayer by scrapping with a sterile pipette tip. The cells were washed with PBS again, followed by treatment with various concentrations of TMP (2 and 10 μM) or TMP (0, 100, 200 and 400 μM) liposomes in 1 ml of DMEM (1% serum) medium and then incubated at 37°C in a humidified 5% CO_2 atmosphere for 24 h. After 24 h, the medium was replaced by fresh conditioning medium, followed by 24 h incubation. After this treatment, unattached cells were washed out by rinsing with PBS and the attached cells were fixed in 2% paraformaldehyde. The cell migration from the wound edge was visualized under a reverse phase microscope and area of cell migration was analyzed using the Image Pro Plus (Ver 6.0) software. Experiments were done in triplicates, and the representative images were chosen.

2.8 Tube formation assay

The tubular structure of human umbilical vein endothelial cells (HUVECs) was evaluated by means of a tube formation assay (Abdollahi et al., 2003). Matrigel (250 μl) (10 mg/ml) (BD Biosciences, New Jersey) was polymerized for 30 min at 37˚C. HUVECs were suspended in M199 (5% FBS) medium (Sigma Chemical Co., St Louis, MO, USA) at a density of 2.5×10^5 cells/ml, and 0.2 ml of cell suspension was added to each well that had been coated with Matrigel, together with the indicated concentrations of TMP or TMP liposomes, followed by incubation at 37°C in a humidified 5% CO_2 atmosphere for 14 h. Morphological changes of the cells and HUVEC tubes formation were observed under a phase-contrast microscope and photographed at ×40 magnification.

2.9 *In vivo* anti-metastatic activity studies

To evaluate the anti-metastatic effect of TMP and TMP liposomes, two lung metastasis models, *i.e.*, experimental metastasis model (intravenous injection of tumor cells) and spontaneous metastasis model (subcutaneous injection of tumor cells), were used following a previous report (Park et al., 1994). The experimental metastasis model was produced by injection of B16F10 murine melanoma cells (5×10^4) in 100 μl serum-free media into the lateral tail veins of C57BL/6 mice. They were administrated with various doses of TMP (0.4 and 2 mM) or TMP (0.4 and 2 mM) liposomes in 200 μl of serum-free media at 15 min, 5 and 10 days after tumor inoculation, respectively. On day 18, the lungs were harvested and fixed in 2% paraformaldehyde solutions after the animals had been sacrificed with carbon dioxide. The colony numbers in the lungs were compared after dissection to facilitate the measurements. The spontaneous metastasis model was produced by injection of B16F10 cells (2×10^5) in 40 μl of serum-free media into the footpad (subcutaneous injection) of C57BL/6 mice, and TMP (2 mM) or TMP (2 mM) liposomes were administrated on days 5, 10, 15, 20 and 25 after tumor inoculation, respectively. The primary tumors in feet of mice were excised on day 21. After the animals were sacrificed with carbon dioxide on day 38, the lungs were harvested and fixed in 2% paraformaldehyde solutions. The numbers of colonies in lungs were compared after dissection to facilitate the measurement. These experiments were approved by the Animal Care and Use Committee of the College of Pharmacy, Seoul National University.

2.10 The measurement of Protein Kinase C (PKC) activity

PKC activity was determined by using CycLex® protein kinase C assay kit (CycLex Co., Nagano, Japan). The assay procedures were performed according to CycLex® PKC assay user manual. The PKC inhibitory effect of TMP was measured in various concentrations of TMP (10, 25, 50 and 100 μM).

2.11 Western blot analysis

Whole-cell extracts of B16F10 cells, which were incubated with various concentrations of TMP (2, 10 and 25 μM) or TMP (100, 200 and 400 μM) liposomes for 24 h, were prepared in radioimmunoprecipitation assay (RIPA) buffer (Thermo Scientific, Rockford, IL, USA) containing protease inhibitors (Thermo Scientific, Rockford, IL, USA). Protein concentrations were then determined using a bicinchoninic acid (BCA) protein assay kit following the manufacturer's instructions (Sigma Chemical Co., St Louis, MO, USA). Samples (50 μg of total protein) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., MA, USA). Protein transfer was checked by Ponceau S solution staining (Sigma Chemical Co., St Louis, MO, USA). After blocking with 5% skim milk in phosphate buffered saline, the blots were incubated with specific antibodies including VEGF (Novus Biologicals, Littleton, CO, USA), MMP-2 (Santa Cruz Biotechnology, Inc., CA, USA), and β-actin (Abcam, Cambridge, UK), followed by the secondary antibody conjugated to horseradish

peroxidase and detected by an enhanced chemiluminescence (ECL) reagent. The secondary antibodies and ECL reagent were purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.12 Statistics

Values were presented as the mean ± standard deviation (S.D.). The Student's t-test was used to determine statistical significance between pairs of samples. A p-value below 0.05 was considered to be significant.

3. Results

3.1 Effects of TMP and TMP liposomes on cytotoxicity and hemolysis

The IC_{50} values for TMP or TMP liposomes on five-cancer cell lines are summarized in Table II. Significantly lower cell cytotoxicity was observed for TMP liposomes than for TMP on all five-cancer cell lines. Additionally, the sensitivity of B16F10 murine melanoma cells was lower compared to the other cell lines. These results indicate that, although TMP itself exhibits a severe cytotoxicity, liposomal formulations effectively reduce the cytotoxicity on both human and murine cancer cell lines. B16F10 cell line was used to evaluate the anti-metastatic effect of TMP and TMP liposomes due to their highly invasive malignant properties as well as their lower cytotoxic sensitivity compared to other cell lines. To verify whether antitumor effects of TMP were due to apoptosis, Annexin V/PI assay was performed after treatment with TMP and TMP liposomes for 6 h and 12 h, respectively (Fig 3A and 3B). As shown in Figure 3, TMP increased the necrosis quadrant in correlation to increased concentration of TMP, and almost induced necrosis at 50 μM TMP. But the necrosis quadrant after treatment with TMP liposomes (400 μM TMP) for 12 h was slightly increased in comparison with blank liposomes.

After inoculating mice with high concentrations of TMP solutions (2 mM) through the tail vein, TMP induced hemoglobinuria and a local inflammation response (data not

shown). As shown in figure 4, TMP did not induce hemolysis at concentrations of up to 100 μM, but erythrocytes were completely lysed in concentrations of 1 mM TMP and above. A hemolytic effect of TMP at concentrations of up to 2 mM was not observed in the case of treatment with TMP liposomes.

3.2 Inhibition of cell migration by TMP or TMP liposomes

Cell migration in metastasis, tissue invasion and cancer cell scattering are important markers for evaluating malignant tumors. The migration of B16F10 melanoma cells was investigated using the scratch wound assay after treatment with TMP or TMP liposomes. Fig. 5A shows the morphology of the B16F10 cell monolayers after scraping with a sterile pipette (0 day), and those after 2 days of treatment with 10 μM TMP, liposomes containing 400 μM TMP in DMEM media and no treatment (control). After 48 hours, B16F10 cells cultured in the absence of TMP (control and 0 μM TMP liposomes) or in the presence of 2 μM TMP solution had recolonized nearly 100% of the wound. However, B16F10 cells cultured in the presence of 10 μM TMP solution as well as 100, 200 and 400 μM TMP liposomes showed a significantly reduced wound healing of about 40 to 50% (Fig. 5B).

3.3 Reduction of tube formation

The production of new blood vessels is an essential process in angiogenesis. Therefore, the inhibitory effects of TMP or TMP liposomes on HUVEC tube formation

were evaluated. As shown in Figure 6, HUVECs that had been treated with the TMP solution formed tubular structures up to a concentration of 10 μM, although the formation of capillary networks was slightly reduced. When HUVECs were incubated with TMP solutions at concentrations above 25 μM, no capillary-like network could be detected. HUVECs treated with TMP liposomes containing levels of TMP up to 200 μM formed capillary-like networks. But, the luminal structure of HUVECs treated with TMP (400 μM) liposomes was morphologically altered and formed fewer as well as weaker tubes when compared to that of HUVECs treated with TMP (0 μM) liposomes.

3.4 The effect of TMP and TMP liposomes on *in vivo* lung metastasis

As mentioned above, TMP inhibits tumor migration in B16F10 melanoma cells and tube formation in HUVECs, and the efficacy of the inhibition is dependent on the concentration of TMP. The issue of whether TMP inhibits tumor metastasis was also examined. To investigate the efficacy of TMP and TMP liposomes in an *in vivo* lung metastasis model, B16F10 cells were utilized. In experimental metastasis (Fig. 7), the median number of lung nodules after treatment with TMP (400 μM) or TMP (400 μM) liposomes was not significantly different from that of PBS. The number of lung nodules after treatment with TMP (2 mM) liposomes was significantly reduced comparison with that of PBS, and treatment with TMP (2 mM) resulted in slight reduction in the number of nodules compared to that of PBS. For the treatment of spontaneous metastasis, TMP (2 mM) or TMP (2 mM) liposomes were injected through tail vein after inoculation of the footpad in mice with tumor cells. Both TMP (2 mM) and TMP (2 mM) liposomes

significantly suppressed tumor metastasis compared to that of PBS and TMP (0 μM) liposomes (Fig. 7). In addition, no notable side effects were observed for the TMP (2 mM) liposomes, while severe local tissue damage was clearly observed when TMP (2 mM) solutions were used.

3.5 Cellular mechanism of TMP

The previous results showed that TMP and TMP liposomes inhibit not only tumor migration and the formation of tubular structures *in vitro*, but also *in vivo* lung metastasis. The activation of PKC-α promotes angiogenic activity of human endothelial cells. Moreover this is correlated with induction of VEGF (Xu et al., 2008). In order to inhibition effect of PKC on TMP, PKC activity was examined after TMP treatment. As shown in Figure 8, PKC activity was inhibited above 50 μM TMP. In addition, effect of TMP in pre-angiogenesis factor was evaluated. Vascular endothelial growth factor (VEGF) is a major signal protein that induces not only angiogenesis, but also vasculogenesis (Kim et al., 1993). MMP-2 is also associated with various cell behaviors, including cell migration and angiogenesis (Sang, 1998; Zheng et al., 2006). To better understand these relationships, it was determined by western blotting whether TMP or TMP liposomes inhibited the expression of VEGF and MMP-2. Western blot analysis showed that incubation with TMP and TMP liposomes inhibited the activity of VEGF and MMP-2 (Fig. 9). Moreover the expression of VEGF and MMP-2 was dependent on the concentration of TMP.

4. Discussion

Since the tumor metastasis is closely associated with angiogenesis, the focus of much of the current cancer research has been on anti-angiogenic compounds (Folkman, 1971; Rosen, 2000). Sphingolipids and their metabolites have been extensively studied as important mediators of cellular processes. Previous studies demonstrated that phytosphingosine derivatives induce apoptotic cell death through various cell signaling pathways (Kim et al., 2009; Park et al., 2003; Park et al., 2010). Nevertheless, the physiological role of TMP, which is a phytosphingosine derivative, in tumor metastasis remains largely unknown. In this report, anti-angiogenic activities such as the inhibition of proliferation and cell migration and the suppression of tube formation by TMP were explored. The results show that 10 μM TMP significantly suppresses the migration of B16F10 melanoma cells compared to the control (Fig. 4). The findings also show that TMP inhibits the formation of tubular structures at concentrations above 25 μM (Fig. 5). Although it is known that TMP plays an important role in tumor anti-angiogenic activity, its clinical application was difficult due to side effects including severe cell cytotoxicity, hemolysis and local inflammation in mice after intravenous injection (preliminary experiment, data not shown). Therefore, utilizing the well-known ability of liposomal formulations to enhance cellular uptake and reduce side effects (Gabizon et al., 1982, 1995; Matsuo et al., 2001), liposomes was used as a delivery carrier for clinical trials in an attempt to inhibit tumor metastasis by TMP.

The anti-angiogenic activities of TMP and TMP liposomes were evaluated, and it

was found evident that they successfully inhibit cell migration and tube formation (Fig. 4 and 5). Additionally, TMP liposomes inhibited the migration of B16F10 cells in a concentration-dependent manner up to a TMP concentration of 400 μM, and no negative effects on culture growth were observed. These results show that TMP and TMP liposomes effectively suppress angiogenic activities, and have inhibitory effects on *in vivo* tumor metastasis. In experimental tumor metastasis, the number of lung colonies in mice after intravenous administration of TMP (400 μM) or TMP (400 μM) liposomes was not significantly different compared to that of the control. However, TMP (2 mM) liposomes significantly suppressed the formation of lung colonies in mice when compared to the control, while TMP (2 mM) solution reduced lung colony formation, however not significantly (Fig. 6). This may be due to the fact that the suppression of metastasis by TMP or TMP liposomes was insufficient in experimental tumor metastasis model since tumor cells were directly introduced into the bloodstream *via* intravenous administration (Park et al., 1994). On the other hand, both TMP (2 mM) and TMP (2 mM) liposomes significantly reduced the number of lung colonies in mice in the spontaneous tumor metastasis model (Fig. 7). Since tumor cells were injected into the foodpads by subcutaneous injection, TMP could show the inhibition of metastatic effect more efficiently. Although it is also interesting to note that inhibitory effects on tumor metastasis by TMP and TMP liposomes were not significantly different at the 2 mM concentration. *In vivo* administration of TMP (2 mM) solution was not feasible due to undesirable side effects such as local thrombophlebitis and severe tissue damage as well as hemoglobinuria caused by hemolysis (data not shown), while TMP liposome did not show any notable side effects at the same concentration. Thus, a liposomal formulation

could be a suitable system for delivering a high dosage of TMP and successful inhibit metastasis.

TMP might slightly offset the cell-signaling pathways of angiogenesis and inhibit metastasis in a highly metastatic B16F10 cell line. Previous studies suggested that angiogenesis is related to PKC acitivity and various cancer cell lines are able to secrete pro-angiogenic factors such as VEGF and MMP-2. Thus, we investigated whether TMP and TMP liposomes could counteract the PKC and angiogenic factors of B16F10 cells. It was observed that 100 μM of TMP could inhibit PKC activity up to 40% (Fig. 8). In the western blot assay, it was found that TMP and TMP liposomes were able to slightly suppress the expression of VEGF and MMP-2 by B16F10 cells (Fig. 9). These results also indicate that the inhibitory effects of TMP on PKC, VEGF and MMP-2 expression are concentration-dependent.

5. Conclusion

The results of this study indicate that TMP exhibits significant anti-angiogenic and metastatic activities on B16F10 cells, and that this occurs by reducing PKC activity and the expression of angiogenic factors such as VEGF and MMP-2. However, TMP has undesirable side effects and low *in vivo* anti-metastatic activities. The use of liposomal carriers can deliver high dosage of TMP (up to 2 mM) with reduced cytotoxicity and side effects of TMP while at the same time these delivery systems can enhance the anti-angiogenic and metastatic activities of TMP both *in vitro* and *in vivo*. The findings reported here suggest that TMP is a potential inhibitor of tumor angiogenesis as well as metastasis, and that liposomal formulation of TMP can be an effective delivery system for this compound.

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Table I. Physical properties of TMP liposomes

Concentration of TMP (μM)	Size (nm)	Poly dispersity	Zeta potential (mV)
0	155.16±22.45	0.17±0.07	-2.97±5.77
400	152.23±7.27	0.12±0.05	-0.27±4.84

Table II. IC_{50} of TMP and TMP liposomes

Name	Origin	Species	TMP IC_{50} (μM)	TMP Liposomes IC_{50} (μM)
HepG2	Liver	Human	26.61±1.93	>400
MCF7	Breast	Human	14.42±0.58	>400
A549	Lung	Human	15.28±0.50	>400
A375P	Melanoma	Human	12.97±0.49	372.46±42.51
B16F10	Melanoma	Murine	33.34±1.03	>400

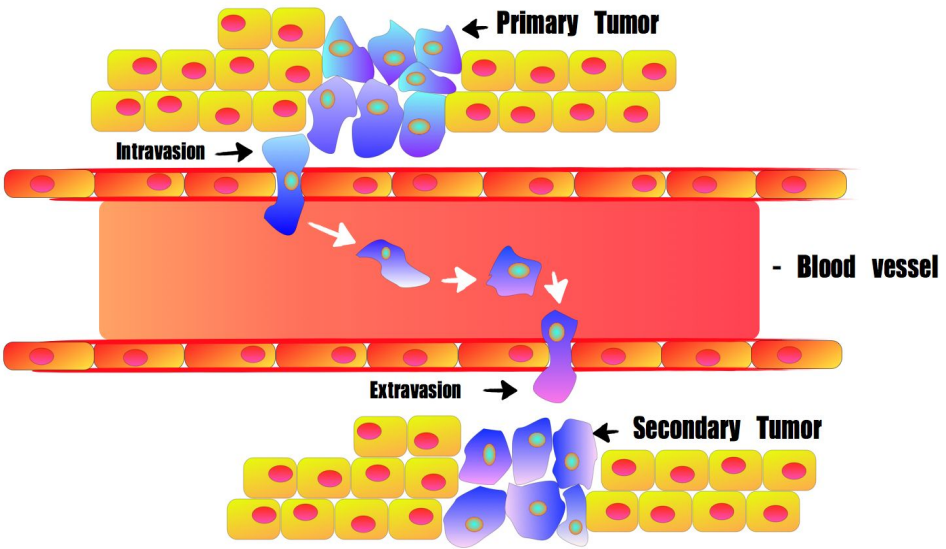


Figure 1. The principle of tumor metastasis

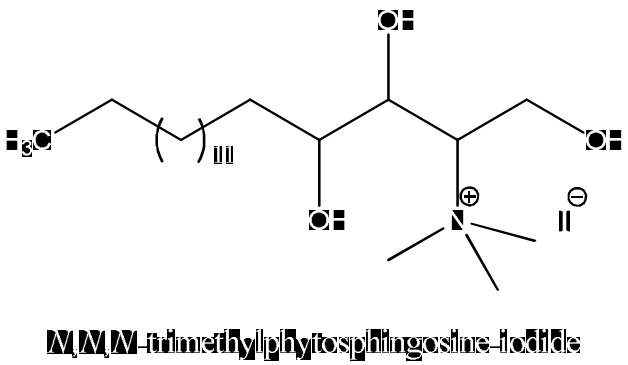
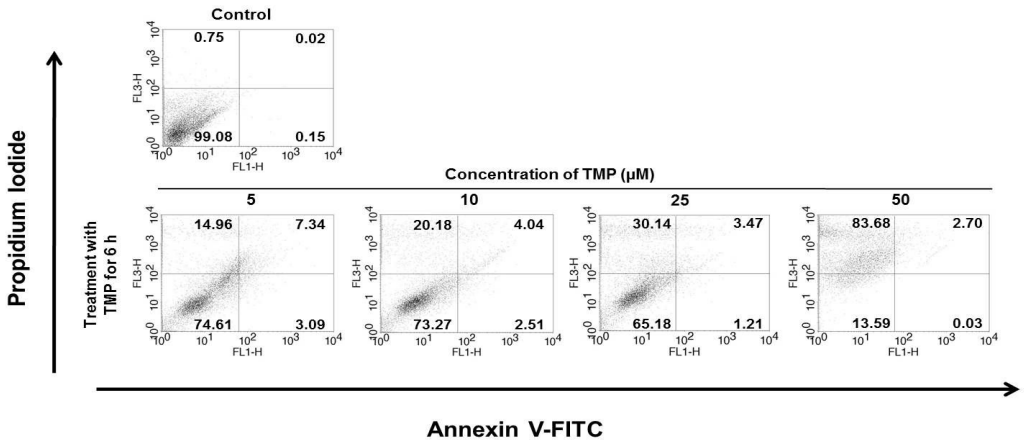


Figure 2. Chemical structure of *N,N,N*-trimethylphytosphingosine-iodide (TMP)

A



B

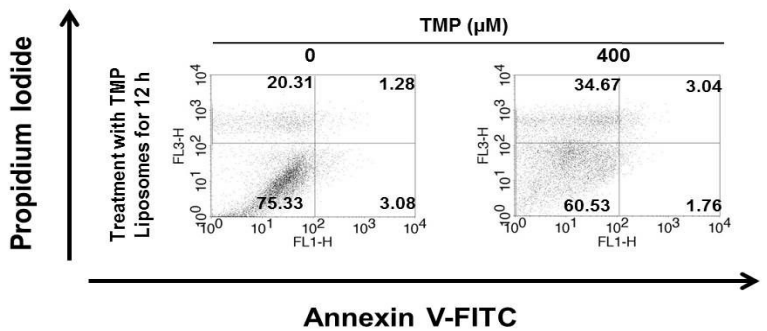


Figure 3. Induction of apoptosis in B16F10 cells after treatment TMP (A) and TMP liposomes (B). Various concentration of TMP or TMP liposomes treated B16F10 cells for 6 h or 12 h, respectively. Representative data are shown the numbers represent percent cells in the appropriate quadrant. (Left bottom: viable cells; Right bottom: early apoptotic cells; Left top: necrosis; Right top: late apoptotic cells).

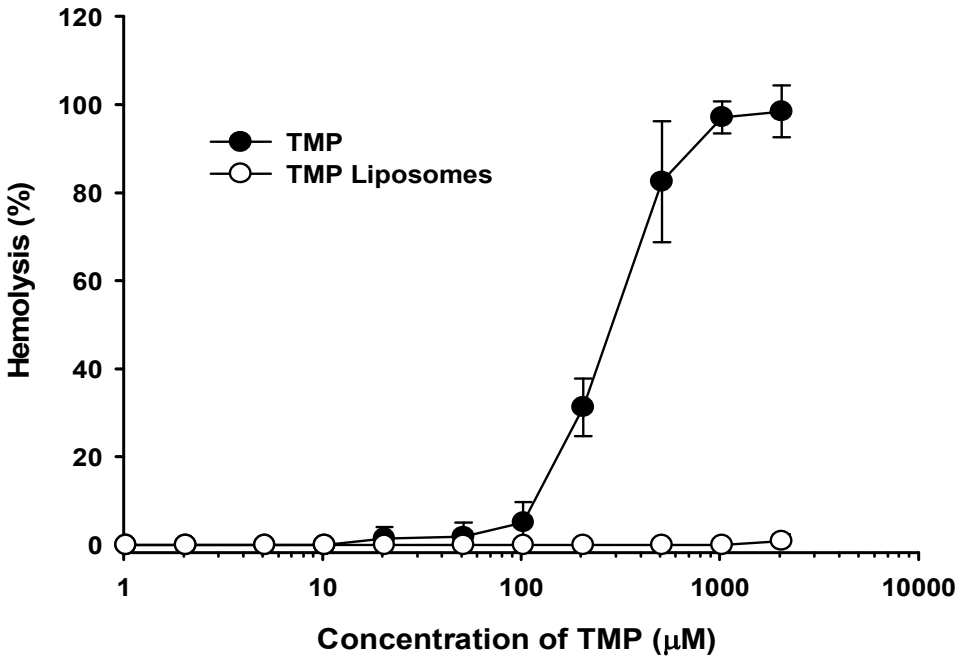


Figure 4. TMP-induced hemolysis. Heparin-treated rat blood was incubated with various concentrations of TMP (●) or TMP liposomes (○) at 37°C for 30 min. The hemolysis percentage was determined by measuring the amount of hemoglobin released at 405 nm.

A

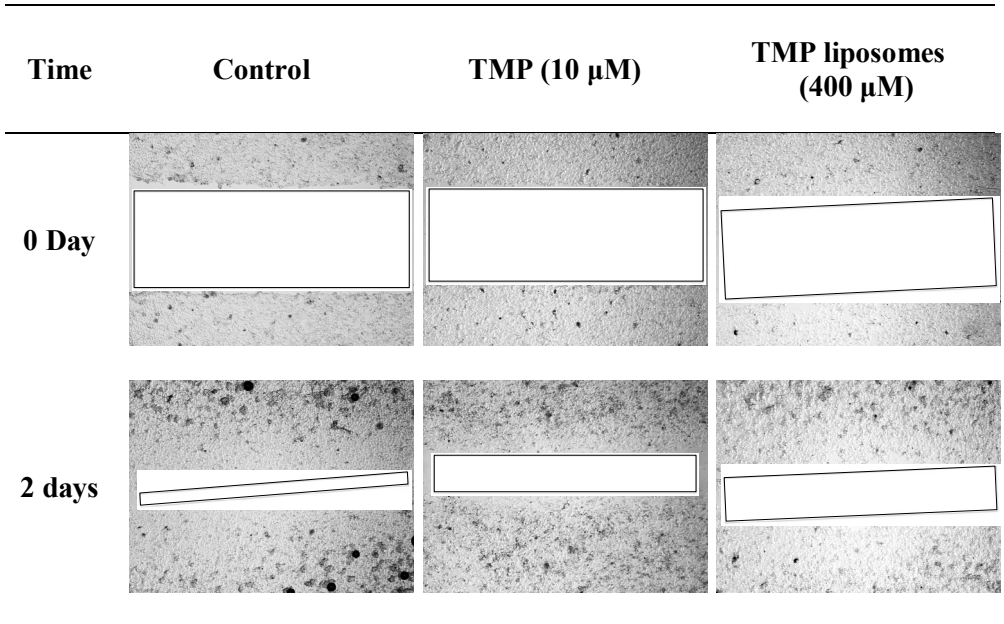


Figure 5. *In vitro* cell migration studies. B16F10 cells (1×10^5 cells/well) were cultured reaching 90% confluence. The cells were incubated with various concentrations of TMP (TMP or TMP liposomes) for 2 days after creating a scratch on the cell monolayer by scraping with a sterile pipette tip. (A) Wound edge morphology was measured using reverse-phase microscopy (magnify 100 times).

B

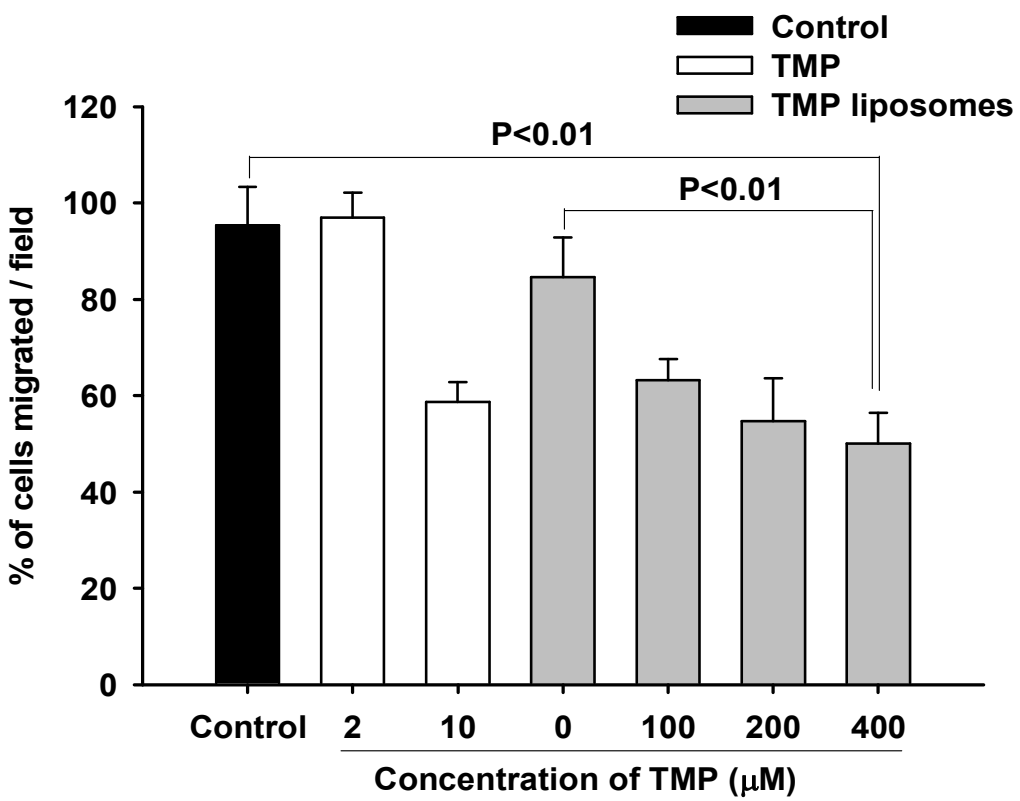


Figure 5. (B) The percent cell migration / field was evaluated from the cell migration area after 2 days

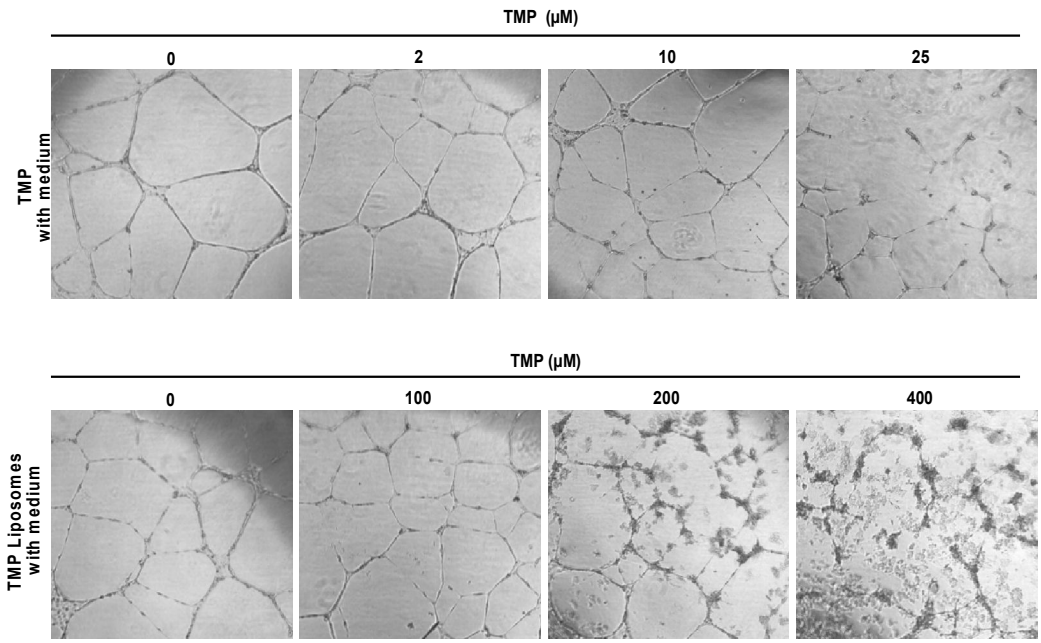


Figure 6. Behavior of endothelial cells (HUVECs) after treatment with TMP (2, 5, 10 and 25 μM) or TMP liposomes (0, 100, 200 and 400 μM TMP)

A

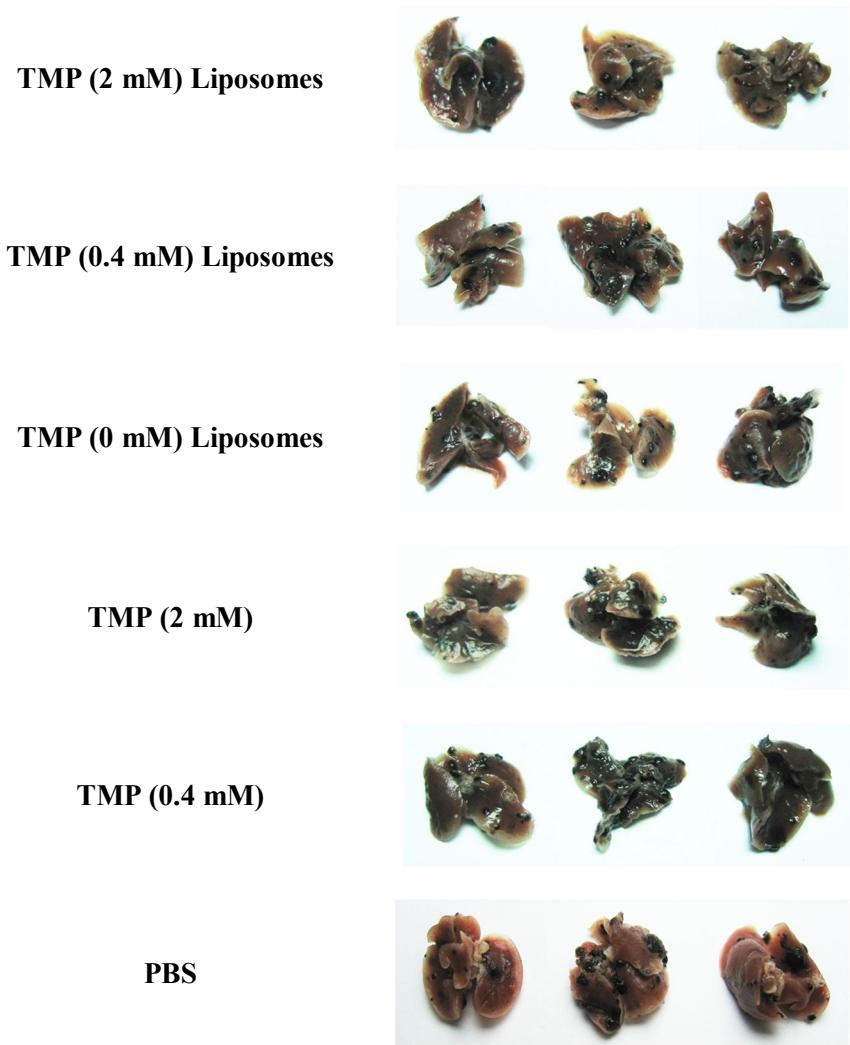


Figure 7. Inhibitory effect of TMP and TMP liposomes in treating experimental metastasis. TMP or TMP liposomes were injected intravenously at 15 min, 5 and 10 days after inoculation of B16F10 cells via the tail vein in mice. Numbers of lung colonies were counted on day 18. (A) Image for metastatic colonies on the lungs (representative images).

B

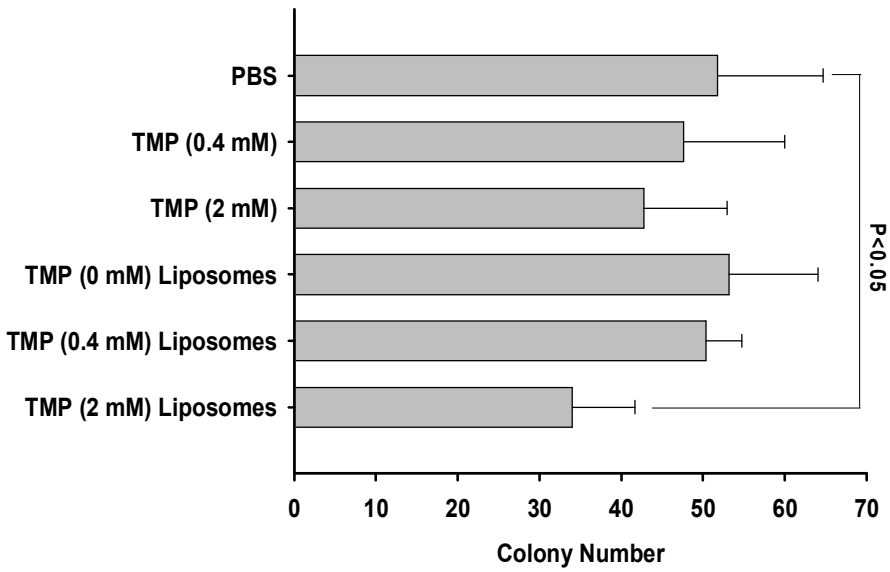


Figure 7. (B) The number of metastatic colonies on the lung. The data represent the mean ± S.D. (n=5)

A

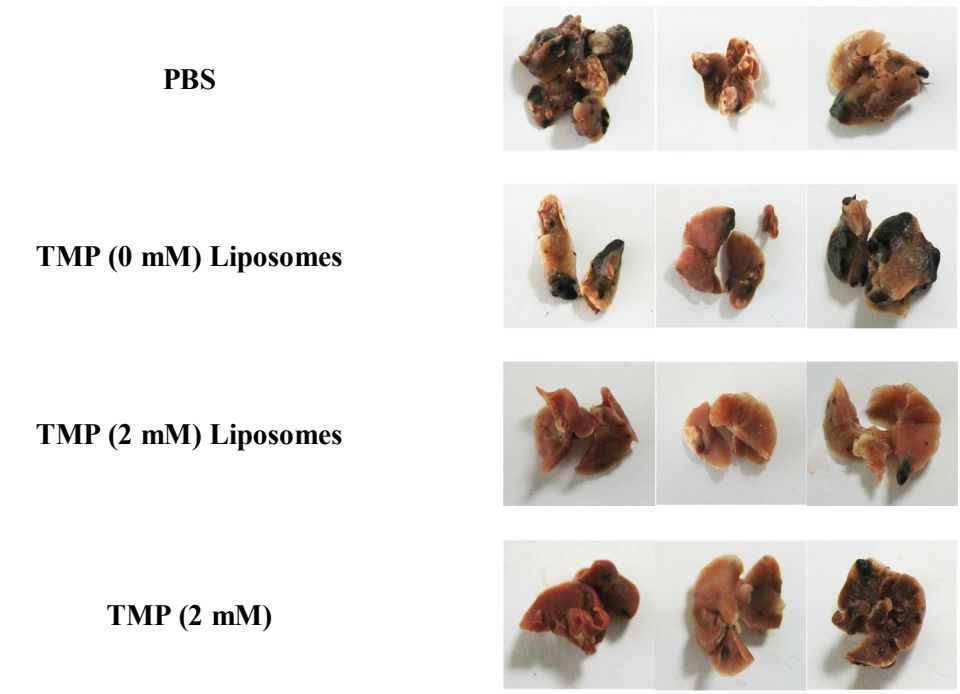


Figure 8. Inhibitory effect of TMP and TMP liposomes in treating spontaneous metastasis. TMP or TMP liposomes were injected intravenously at 5, 10, 15, 20 and 25 days after inoculation of B16F10 cells via the footpad in mice. Lung colonies were counted on day 38. (A) Image for metastatic colonies on the lungs (representative images).

B

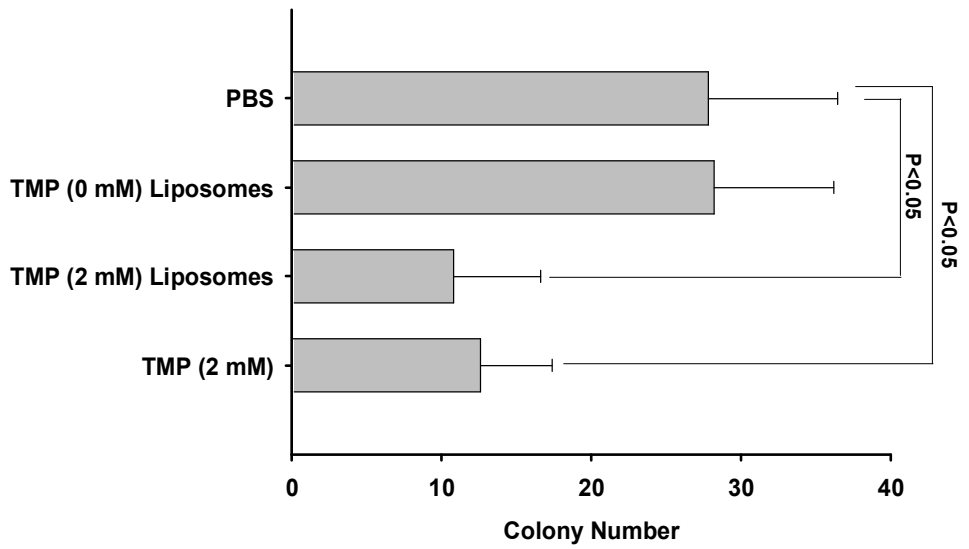


Figure 8. (B) The number of metastatic colonies on the lung. The data represent the mean ± S.D. (n=5)

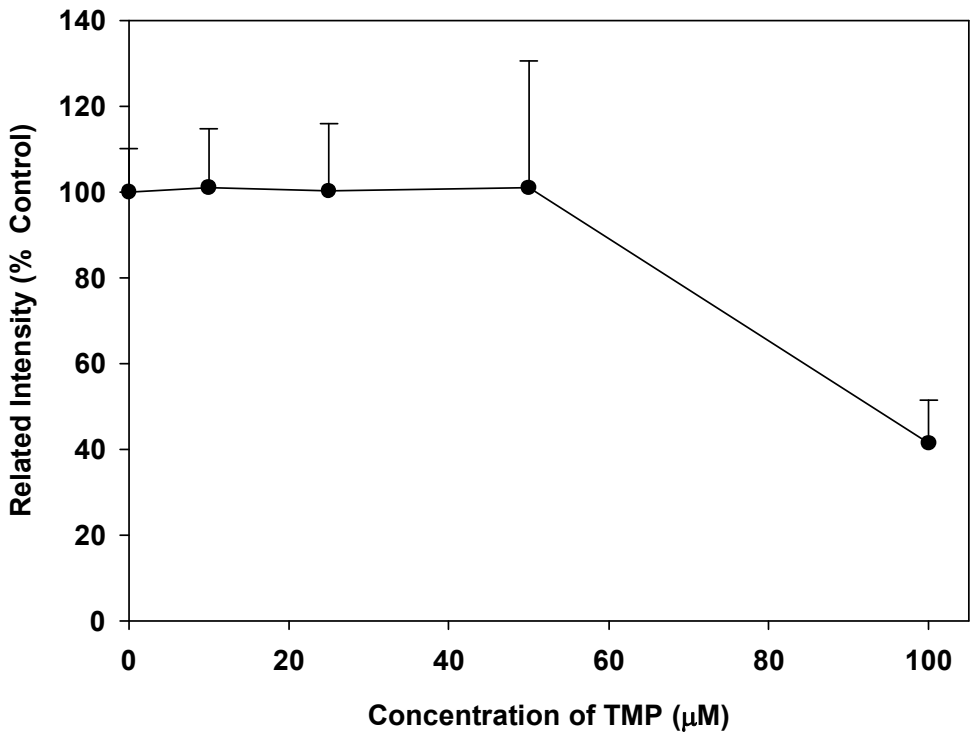
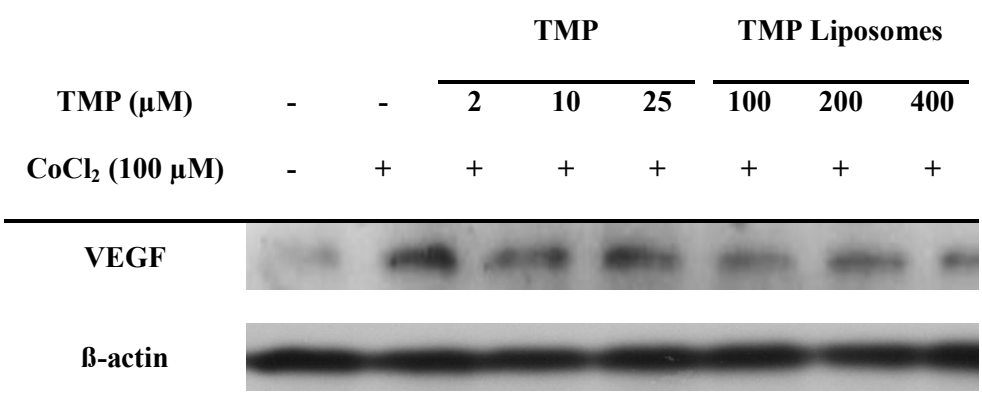


Figure 9. Inhibitory effect on PKC activity after treatment of various concentration TMP

A



B

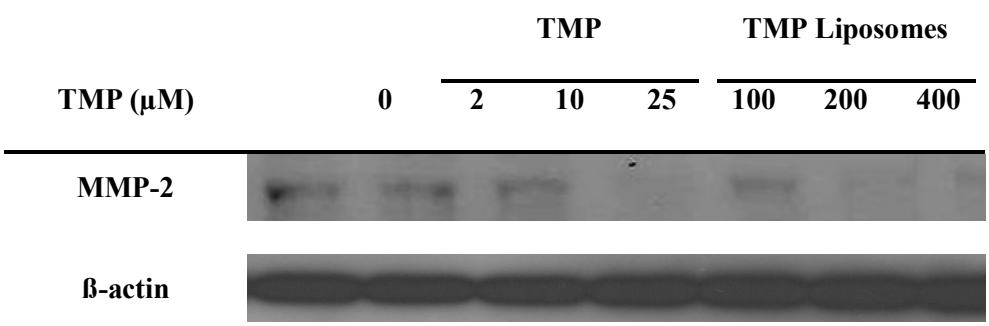


Figure 10. Expression of VEGF (A) and MMP-2 (B) in B16F10 melanoma cells after treatment with TMP or TMP liposomes for 24 h, as determined by western blot

국문초록

새로운 혈관이 생성되는 과정인 혈관신생은 상처치유 및 성장등에 있어서 필수적인 생리적 과정이지만, 종양의 성장에 있어서도 필수적인 과정으로 알려져 있다. 종양에서의 혈관 신생은 종양의 성장에 있어서 필요한 영양분의 공급역할을 할뿐만 아니라 혈관계를 통하여 다른 조직으로 종양세포가 전달되는 전이에도 중요한 역할을 한다. 따라서 혈관신생 억제 물질을 통한 치료방법은 암치료에 있어서 관심이 집중되고 있으며 혈관신생억제에 의한 전이치료에 있어서도 관심이 집중되고 있다. 지질의 한 종류인 세라마이드, 스핑고신 그리고 피토스핑고신등과 같은 스핑고지질은 세포의 성장, 분화, 노화 그리고 사멸등의 중요한 mediator로 알려져 있다. 그중 피토스핑고신이나 피토스핑고신의 메틸유도체는 활성산소 (ROS) 생성, 카스파제 (caspase) 활성화 그리고 Bax 전위등과 같은 과정을 통하여 암세포의 자연적 사멸을 유도할 수 있다는 것이 알려져 있다. 그러나 피토스핑고신의 메틸유도체인 N,N,N-트리메틸피토스핑고신(N,N,N-trimethylphytosphingosine- iodide, TMP)의 혈관신생 및 전이 억제에 대해서 보고되지 않았다. 또한 스핑고지질을 임상실험에 적용시 용혈, 혈색소뇨 그리고 투여부위의 염증반응과 같은 부작용이 존재하는 것으로 알려져 있어, 이를 안전하게 전달할 수 있는 약물전달시스템이 필수적이다. 따라서 이번 연구에서는 in vitro/in vivo 내에서의 TMP에 의한 혈관신생 및 암전이 억제에 대한 연구를 진행하였으며, TMP를 안전하고 효과적으로 전달할

수 있는 리포솜을 이용한 약물전달시스템을 개발 및 평가하였다.

본 연구에서는 TMP의 부작용을 줄이고 혈관신생 및 암전이를 억제할 수 있는 실험을 진행하기 위하여 TMP를 포함하는 대략 150 nm 크기의 리포솜(TMP-리포솜)을 제조하였으며, TMP와 TMP-리포솜에서의 세포독성 평가를 통하여 B16F10 마우스 유래 흑색종 세포를 선정하였다. 스핑고신의 대표적인 부작용인 용혈현상이 TMP에서 나타나는지 확인하기 위하여 Rat whole blood를 이용하여 TMP의 용혈현상을 평가한 결과 100 μM이상에서 용혈현상이 관찰되었으며 500 μM에서 80%이상의 혈액이 용혈되었다. TMP-리포솜을 이용하여 용혈현상을 평가한 결과 TMP 용액과 비교하여 부작용이 크게 줄어들었으며 2 mM TMP가 포함된 리포솜까지 용혈현상은 관찰되지 않았다. 혈관신생 평가인 cell migration과 HUVECs tube formation 실험에서 TMP와 TMP-리포솜은 TMP 농도 의존적으로 cell migration과 혈관신생을 억제하는 것을 확인하였다. 위의 in vitro 혈관신생 억제효과가 암전이에 효과가 있는 것을 확인하기 위하여 experimental 과 spontaneous metastasis model을 B16F10 세포주를 이용하여 마우스에 확립 한 후 TMP용액과 TMP-리포솜을 투여시 TMP 농도 의존적으로 마우스 lung colony의 수가 줄어드는 것을 확인할 수 있었다. TMP-리포솜의 경우 마우스의 꼬리 미정맥에 투여시 부작용이 관찰되지 않은 반면에 TMP 용액의 경우 투여부위에 용혈 및 염증반응과 같은 부작용이 확실하게 관찰되었다. TMP가 혈관신생과 암전이 억제에 효과가 있는 것으로 결과를 확인 후 혈관신생 인자인 VEGF와 MMP2의 발현정도를 western blot assay를 통하여 확

인한 결과 TMP와 TMP-리포솜 모두 TMP농도 의존적으로 발현정도가 감소되는 것을 확인할 수 있었다.

결론적으로 스핑고지질 유도체인 TMP가 VEGF와 MMP-2를 감소시킬 수 있는 물질로서 혈관신생과 암전이 억제에 있어 효과적으로 사용될 수 있다. 그러나 TMP를 임상적으로 적용함에 있어 발생할 수 있는 용혈 및 염증등과 같은 부작용을 줄이기 위해 리포솜을 이용한 TMP-리포솜을 제조하였다. 이러한 리포솜시스템은 TMP 단일사용시 발생하는 부작용을 줄일 수 있으며 TMP의 효과를 유지할 수 있는 효과적인 약물전달시스템으로 적용될 수 있다.

주요어: 트리메틸피토스핑고신, 리포솜, 혈관신생, 전이

학 번: 2007-30462